ORIGINAL ARTICLE

Pharmacodynamics of amoxicillin/clavulanic acid against *Haemophilus influenzae* in an in vitro kinetic model: a comparison of different dosage regimens including a pharmacokinetically enhanced formulation

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Objective To study the pharmacodynamics of amoxicillin/clavulanic acid against different strains of *Haemophilus influenzae* in an in vitro kinetic model. The concentrations used corresponded to human serum levels obtained after 875 mg amoxicillin/clavulanic acid given b.i.d, 500/125 mg amoxicillin/clavulanic acid given t.i.d. and those obtained with a pharmacokinetically enhanced formulation containing 1125/125 mg amoxicillin/clavulanic acid (immediate release) and 875 mg amoxicillin (sustained release) given b.i.d.

Methods Bacteria at an initial inoculum of 10^6 colony-forming units (CFU)/mL were exposed to amoxicillin/clavulanic acid with an initial concentration of approximately 15/3 mg/L, 8/3 mg/L simulating the peak levels in humans achieved after a dose of 875/125 mg and 500/125 mg with a half-life of 1 h. In addition, experiments with a 2000/125 mg pharmacokinetically enhanced formulation of amoxicillin/clavulanic acid given b.i.d were performed. A repeated dose was given at 12 h after the initial dose of 875/125 mg and the pharmacokinetically enhanced formulation or at 8 and 16 h after the dose of 500/125 mg. The experiments were performed in an in vitro kinetic model, which consists of a spinner flask with a filter membrane fitted in between the upper part and the bottom part in order to prevent bacterial dilution. The medium is removed from the culture flask, through the filter, at a constant rate with a pump. Repeated samples were taken at intervals of 1–2 h up to 24 h during the experiments for viable counting. One of the strains of *H. influenzae* was also exposed to a constant concentration corresponding to the peak serum levels obtained after a dose of 500/125 mg.

Results The concentrations of amoxicillin in the in vitro kinetic model were as expected. At the end of the experiment (24 h), there was a tendency for a greater bactericidal effect with 500/125 mg t.i.d, as compared to 875/125 b.i.d., with differences in CFUs between the two dosing regimens of 2.6 $\log_{10}$ CFU for *H. influenzae* LH 2803 and 1.8 $\log_{10}$ CFU for the other clinical strains. However, these differences did not reach statistical significance ($P = 0.075$ and 0.10, respectively). A statistically significant higher bactericidal effect was seen in the experiments with the pharmacokinetically enhanced formulation in comparison with the b.i.d. regimen both at 8, 16 and 24 h and at 8 and 16 h with the t.i.d. regimen. With the new formulation, no regrowth was seen at 24 h, similar to the results obtained with a constant concentration.

Conclusions Neither of the standard dosing regimens of amoxicillin (875/125 mg b.i.d. or 500/125 mg) used in our study, in which the time that the free (non-protein-bound) concentration the MIC (T > MIC) exceeding was less than 50%, was sufficient to achieve a complete bactericidal effect during the first 24 h of treatment. However, a statistically significant difference in bactericidal activity was seen at 8, 16 and 24 h vs. the b.i.d.

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regimen and at 8 and 16 h vs. the t.i.d. regimen with the pharmacokinetically enhanced formulation. This formulation gave a longer T > MIC (73–79%) of amoxicillin even though the concentration of clavulanic acid was only detectable for 45% of the dosing interval, and complete killing of all strains was obtained after 24 h.

Keywords Pharmacodynamics, amoxicillin/clavulanic acid, dosing regimens, *H. influenzae*, in vitro kinetic model

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INTRODUCTION

In the early era of antimicrobials, it was suggested that the concentration of penicillin should be maintained above the minimum inhibitory concentration (MIC) throughout the dosage interval [1]. However, during the clinical development of new β-lactam antibiotics, intermittent dosing with relatively long dosing intervals was shown to be effective despite the rapid elimination of most of these compounds. The optimal dosing regimen for antimicrobial agents is, however, still not fully explored. In general, β-lactam antibiotics are known to exhibit a non-concentration-dependent killing, their efficacy is mainly dependent on the time that the free (non-protein-bound) concentration stays above the MIC (T > MIC) [2–4]. The exact fraction of the dosage interval during which this concentration should be exceeded for optimal clinical efficacy is, however, not known and may vary according to the type of β-lactam antibiotics and the site of infection.

Different dosing regimens for amoxicillin and clavulanic acid are currently used in clinical practice [5]. A new formulation of amoxicillin with clavulanic acid is presently under clinical evaluation [6]. The rationale behind this development is to increase the T > MIC and thus optimize the efficacy against respiratory pathogens, e.g. *Haemophilus influenzae* and *Streptococcus pneumoniae* [6].

The aim of the present investigation was to compare the bactericidal activity of a pharmacokinetically enhanced formulation of amoxicillin/clavulanic acid with the currently available formulations given, either 875/125 mg b.i.d or 500/125 mg t.i.d., against different strains of β-lactamase-producing *H. influenzae* in an in vitro kinetic model.

MATERIALS AND METHODS

Antibiotics

Amoxicillin and clavulanic acid were provided by Astra/Zeneca, Södertälje, Sweden and SmithKline Beecham Pharmaceuticals, Worthing, West Sussex, UK. The antibiotic and clavulanic acid were obtained as reference powders with known potency. Fresh solutions were made prior to each experiment.

Bacterial strains and medium

The strains used in the study included one strain of TEM-1 β-lactamase-producing *H. influenzae* LH 2803 obtained from SmithKline Beecham Pharmaceuticals and clinical isolates of β-lactamase-producing *H. influenzae* (3029, 2012, 1041, 2045) obtained from the Clinical Microbiological Laboratory, Uppsala University Hospital, Sweden. The strains were grown for 6 h at 37 °C in Mueller–Hinton broth (Difco Laboratories, Detroit, MI, USA), supplemented with 30 mg/L of haemin and 1% of IsoVitaleX yielding an inoculum of approximately 10^9 colony-forming units (CFU)/mL.

Minimum inhibitory concentrations (MICs)

The MICs of all strains were performed with E test (amoxicillin/clavulanic acid 2:1; Biodisk, Solna, Sweden).

Concentrations of amoxicillin and clavulanic acid

The concentrations of amoxicillin were determined by microbiological agar diffusion methods, using *Bacillus stearothermophilus* ATCC 3032 as the test organism [7]. A standardized inoculum of

spore suspension was mixed with tryptone–glucose agar, adjusted to pH 7.4 and plates were poured. After drying the plates, 0.01 mL volumes of all samples and standards diluted in Mueller–Hinton broth, supplemented with 30 mg/L of haemin and 1% of IsoVitaleX were placed in agar wells and three parallel determinations were made. The concentrations of clavulanic acid were determined with microbiological agar diffusion method using *Klebsiella pneumoniae* ATCC 29665 as the test organism. In order to determine the zones of clavulanic acid in these latter determinations, the agar plates also contained benzylpenicillin at a concentration of 6 mg/L. After drying the plates, 0.03 mL volumes of all samples and standards were placed in agar wells and three parallel determinations were made. The limit of detection was 0.031 mg/L for amoxicillin and 0.1 mg/L for clavulanic acid. The correlation coefficient for the standard curves was always >0.99 and the coefficient of variation on samples analysed on different days was 9%.

In vitro kinetic model

A previously described in vitro kinetic model was used in these experiments [8,9]. It consists of a spinner flask with a 0.45-μm filter membrane and a prefilter fitted in between the upper and the bottom part. A magnetic stirrer ensures homogeneous mixing of the culture and prevents membrane pore blockage. In one of the side-arms of the culture vessel, a silicon membrane is inserted to enable repeated sampling. A thin plastic tubing to a vessel containing fresh medium is connected with the other arm. The medium is removed from the culture flask, through the filter, at a constant rate with a pump. Fresh sterile medium is taken into the flask at the same rate by the negative pressure built up inside the culture vessel. In the experiments simulating the twice (b.i.d.) and thrice (t.i.d.) daily regimens, the antibiotic was added to the vessel and eliminated at a constant rate according to the first order kinetics $C = C_0 \times e^{-kt}$ where C_0 is the initial antibiotic level, C is the antibiotic level at the time t, k is the rate of elimination, and t is the time elapsing since the addition of antibiotic. In the experiments simulating the concentrations of the pharmacokinetically enhanced formulation compound, an absorption phase of amoxicillin/clavulanic acid was also included. To mimic the concentration of this formulation, amoxicillin was diluted at the rate corresponding to the half-life of clavulanic acid and the incoming medium was supplemented with amoxicillin at a rate corresponding to its longer half-life. In order to obtain this, two vessels containing amoxicillin at adequate concentrations were connected in series to the culture vessel. The apparatus was placed in a thermostatic room at 37 °C during the experiments.

Bacterial killing following exposure to simulated human pharmacokinetics

875/125 mg b.i.d. and 500/125 mg t.i.d. of amoxicillin/clavulanic acid

Bacteria, at an initial inoculum of approximately 10^6 CFU/mL, were exposed to amoxicillin/clavulanic acid with an initial concentration of approximately 15/3 mg/L or 8/3 mg/L, simulating the peak levels in humans achieved after doses of 875/125 mg and 500/125 mg, respectively. The simulated half-life was 1 h. A repeat dose was given at 12 h after the initial dose of 875/125 mg or at 8 and 16 h after the dose of 500/125 mg. Repeat samples were taken at 0, 2, 4, 5, 6, 7, 8, 9, 12, 14, 16 and 24 h during the b.i.d. experiments and at 0, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 20 and 24 h during the t.i.d. experiments for viable counting. The samples were, if necessary, diluted in phosphate-buffered saline and three dilutions of each sample were spread on blood agar plates (Colombia agar base with 5% horse blood) incubated at 37 °C in 5% CO_2 in air and counted after 24 h. The reference strain of *H. influenzae* was investigated four times on different occasions with both dosage regimens. The clinical strains were studied once at each dosing regimen. Control cultures were grown in glass tubes.

2000/125 mg pharmacokinetically enhanced amoxicillin/clavulanic acid b.i.d.

The bacterial strains with an initial inoculum of approximately 10^6 CFU/mL were exposed to amoxicillin/clavulanic acid, simulating human pharmacokinetics of the pharmacokinetically enhanced formulation (see Figure 1). This formulation contains 1125/125 mg amoxicillin/clavulanic acid (immediate release) and 875 mg amoxicillin (sustained release) given b.i.d [6].

A repeat dose was given at 12 h. Repeat samples were taken at 0, 1, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 12, 13, 14.5, 17.5 and at 24 h for viable counting. The

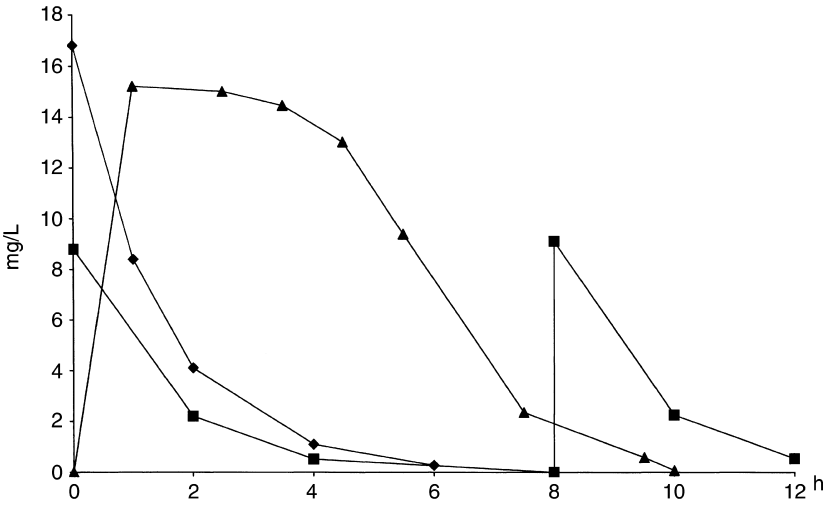


Figure 1 The concentration of amoxicillin in the in vitro kinetic model corresponding to a dose of 875 mg b.i.d. (◆), 500 mg t.i.d. (■) and of the pharmacokinetically enhanced formulation 2000 mg b.i.d. (▲).

samples were cultured as previously described. *H. influenzae* LH 2803 was investigated in triplicate. The clinical strains were studied once. Control cultures were grown in glass tubes.

Static concentrations of amoxicillin/clavulanic acid in the in vitro kinetic model

The rate and extent of killing of amoxicillin/clavulanic acid against *H. influenzae* LH 2803 was also investigated with a constant antibiotic concentration, corresponding to the peak serum levels obtained after a dose of 500/125 mg of amoxicillin/clavulanic acid (8/3 mg/L) This was achieved by adding amoxicillin/clavulanic acid to the fresh medium during the experiments. Samples were withdrawn at 0, 2, 4, 6, 8, 10 and 24 h and seeded and counted as described before. Four experiments were performed on different occasions.

Statistics

The student's *t*-test for unpaired samples was used to compare the bactericidal activities after 24 h of 825/125 mg b.i.d with 500/125 mg t.i.d of amoxicillin/clavulanic acid and to compare the pharmacokinetically enhanced formulation with the b.i.d. and t.i.d. regimens at 8, 16 and 24 h.

RESULTS

Minimum inhibitory concentrations

The MIC value for amoxicillin/clavulanic acid was 0.5 mg/L for *H. influenzae* LH 2803. The remaining four strains had a MIC of 0.75 mg/L

Simulated pharmacokinetics of amoxicillin and clavulanic acid

The concentrations of amoxicillin 875 mg, 500 mg and the concentrations achieved with the pharmacokinetically enhanced formulation are shown in Figure 1. The concentrations of clavulanic acid were as expected and covered 45% of the 24 h with the b.i.d regimen and 69% of the t.i.d. regimen. Table 1 shows the pharmacokinetic/

Table 1 Pharmacodynamic parameters of the different dosages of amoxicillin

	825 mg b.i.d.		500 mg t.i.d		2000 mg b.i.d.	
Strain	C_{max}/MIC	T > MIC_{24}	C_{max}/MIC	T > MIC_{24}	C_{max}/MIC	T > MIC_{24}
H. influenzae LH 2803	33.6	42%	17.6	50%	30.4	79%
H. influenzae 3029, 2012, 1041, 2045	22.4	38%	11.7	44%	20.3	73%

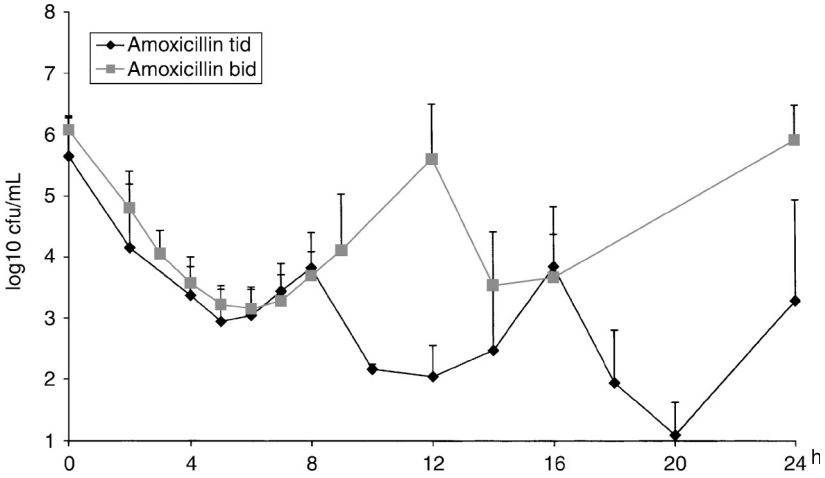


Figure 2 The rate and extent of killing of amoxicillin/clavulanic acid at a concentration corresponding to a dose of 875/125 mg b.i.d. (■) and 500/125 mg t.i.d. (◆) against *H. influenzae* LH 2803 in the in vitro kinetic model (mean ± SD).

pharmacodynamic indices for the different dosing regimens.

Bacterial killing following exposure to simulated human pharmacokinetics

875/125 mg b.i.d and 500/125 mg t.i.d of amoxicillin/clavulanic acid

After exposure to concentrations simulating those obtained with an oral dose of 875/125 mg and 500/125 mg amoxicillin/clavulanic acid, a bactericidal effect of 3–4 $\log_{10}$ CFU/mL was seen initially. This was noted for all the investigated strains. Repeated doses after 8 and 16 h (t.i.d. regimen) exerted a further bactericidal effect. At the end of the experiment (24 h), there was a tendency towards a lower bacterial count with the t.i.d regimen in comparison with the b.i.d. regimen with differences in CFUs of 2.6 $\log_{10}$ CFU and 1.8 $\log_{10}$ CFU for *H. influenzae* LH 2803 (Figure 2) and the other clinical strains, respectively (Figure 3a,b). However, these differences did not reach statistical significance ($P = 0.075$ and 0.10, respectively).

2000/125 mg pharmacokinetically enhanced amoxicillin/clavulanic acid b.i.d.

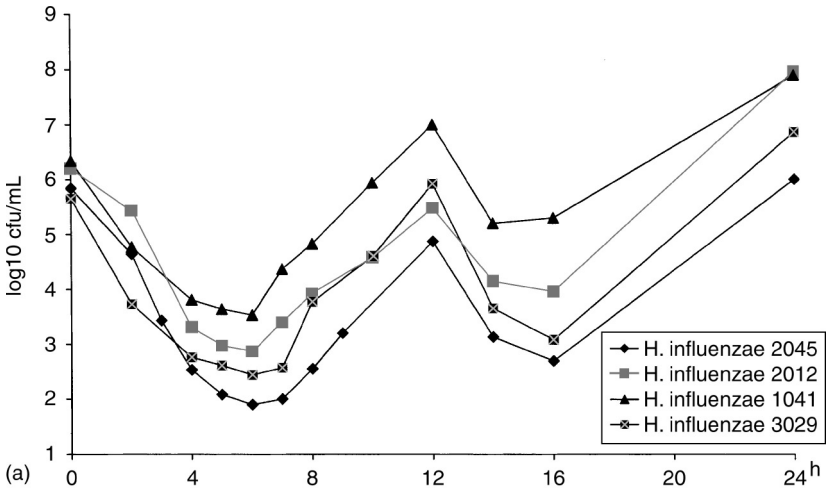
In the experiments simulating the pharmacokinetically enhanced formulation, a bactericidal effect of 4–5 $\log_{10}$ CFU/mL was obtained initially in all experiments. There was a statistically greater bactericidal effect of the pharmacokinetically enhanced formulation in comparison with the standard b.i.d regimen at 8, 16 and 24 h ($P < 0.01$) and at 8 and 16 h ($P < 0.01$) in comparison with the t.i.d. regimen. In the experiments with the pharmacokinetically enhanced formulation, no regrowth was seen except for one of the clinical strains (Figure 4). This strain started to grow between 8 and 9 h but after the second dose at 12 h, a bactericidal effect was obtained and no further regrowth was noted. This was confirmed in two additional experiments.

Static concentrations of amoxicillin/clavulanic acid in the in vitro kinetic model

No regrowth of *H. influenzae* LH 2803 was noted when the bacteria were exposed to amoxicillin/clavulanic acid at a constant concentration of 8/3 mg/L. At 24 h there was a ≥ 5 $\log_{10}$ decrease in viable counts.

DISCUSSION

The goal of antimicrobial therapy is to maximize the bactericidal activity against the infecting pathogen. Results from in vitro studies and studies in animals have shown that different classes of antibiotics behave differently with respect to pharmacodynamic properties. For optimal therapy it is important to take into consideration both pharmacokinetic and pharmacodynamic parameters [2–4]. β-Lactam antibiotics exhibit a minimal concentration-dependent killing, have a short or no post-antibiotic effect or none and it has been shown that the duration of the time that serum concentrations exceeds the MIC (T > MIC) is the important parameter for efficacy [2,10,11]. However, studies in animal models have demonstrated that the antibiotic concentrations do not need to exceed the MIC for the whole dosing interval. Craig et al. have



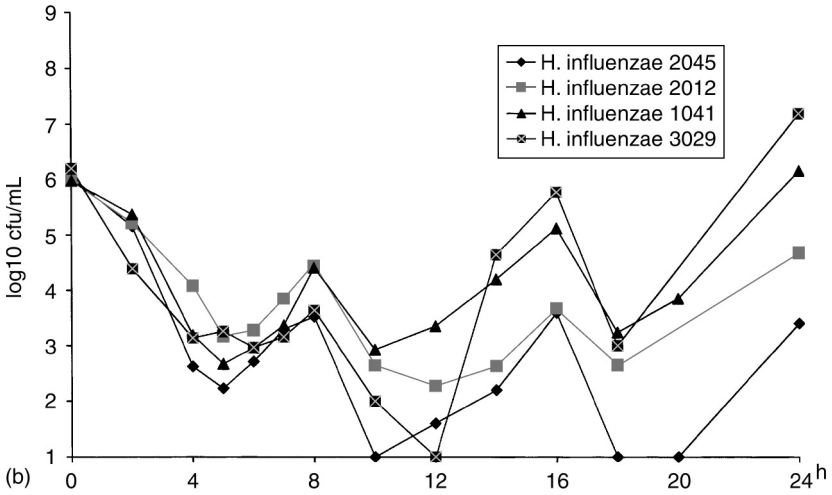


Figure 3 (a) The rate and extent of killing of amoxicillin/clavulanic acid at a concentration corresponding to a dose of 875/125 mg b.i.d. against the clinical strains of *H. influenzae* in the in vitro kinetic model (mean ± SD). (b) The rate and extent of killing of amoxicillin/clavulanic acid at a concentration corresponding to a dose of 500/125 mg t.i.d. against the clinical strains of *H. influenzae* in the in vitro kinetic model.

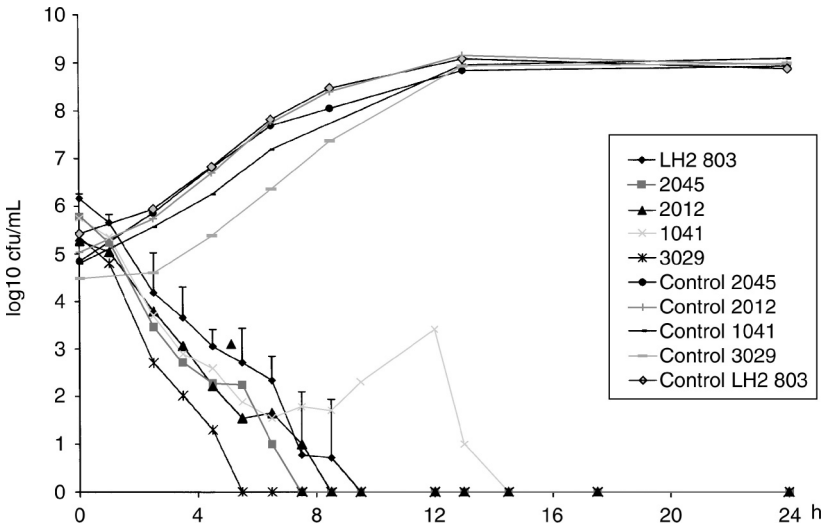


Figure 4 The rate and extent of killing of amoxicillin/clavulanic acid simulating the slow-release compound against *H. influenzae* LH 2803 and the clinical strains of *H. influenzae* in the in vitro kinetic model (mean ± SD).

shown, in thigh and lung infection models in neutropenic mice, that the T > MIC for β-lactam antibiotics against *Streptococcus pneumoniae* needed to be approximately 40–50% of the dosage interval in order to achieve a 90–100% survival of the mice after 4 days of treatment. When the T > MIC was ≤20%, the mortality was almost 100% [3]. In another study in neutropenic mice, by Andes and Craig, the breakpoints of amoxicillin against *S. pneumoniae* were investigated [12]. They also showed that for the mice to survive after 4 days of treatment, a T > MIC of 40% of the 8-h dosing interval was required [12].

Concerning clinical trials, Craig and Andes compiled data retrospectively that included patients with otitis media caused by *S. pneumoniae* and *H. influenzae*, where the microbiological efficacy was followed by repeated tympanocenteses. They found that an increased time during which free serum levels were above the MIC (calculated from published serum pharmacokinetic data in pediatric populations) correlated with an increased bacteriological eradication of the infecting pathogens. To achieve an 80–85% bacteriological cure rate of both cephalosporins and penicillins against the two pathogens, T > MIC of 50% and 40%, respectively, was required [3]. Recently, Dagan et al. have published data on bacterial eradication in the treatment of sinusitis, and found the same figures for T > MIC as with otitis media [4].

In the present study, when simulating the pharmacokinetics following a dose of 875/125 mg of amoxicillin/clavulanic acid b.i.d or 500/125 mg t.i.d, we consistently found regrowth shortly after the concentrations had declined under the MIC and neither dosage regimen was completely bactericidal during this short-term experiment. The mean times for which amoxicillin concentrations exceeded MIC during the 24 h experimental period after a simulated dose of 875/125 mg b.i.d. were 42% and 38% for *H. influenzae* LH 2803 and the clinical isolates, respectively. For the 500/125 mg t.i.d. regimen, the corresponding figures were 50% and 44%. The longer T > MIC in the latter experiments is in accordance with the tendency in our experiments towards a better efficacy with the t.i.d. regimen, although this difference was not statistically significant. It should be noted, however, that an absorption phase, which would have extended the T > MIC somewhat, was not included in these experiments. In the experiments with the pharmacokinetically enhanced formulation, regrowth was only noted for one clinical strain (1041), which started to grow at MIC, but was eradicated after the second dose. The T > MIC in these experiments was 79% for *H. influenzae* LH 2803 and 73% for the other strains with a MIC of 0.75 mg/L. It is notable that the detectable concentration of clavulanic acid in these experiments was only present for 45% of the 24 h. The lack of regrowth despite a non-detectable clavulanic acid concentration may be explained by the post-β-lactamase inhibitor effect [13]. In the experiments with static concentrations, where the T > MIC was 100% during the study period, no regrowth was observed.

Our in vitro model, like others, does not include the potential synergistic effects between the antibiotic and immune defence factors and may thus reflect the situation in an immunocompromised host rather than in a normal patient. Neither of the dosing regimens used in our study, in which T > MIC of amoxicillin was less than 50%, was sufficient to achieve a complete bactericidal effect during the first 24 h of treatment. However, when the concentrations of the pharmacokinetically enhanced formulation were simulated, giving a longer T > MIC of amoxicillin even though the concentration of clavulanic acid was similar to those in the first experiments, complete killing of all strains was obtained after 24 h. Our results are in agreement with others [2–4] in that a better antibacterial efficacy was achieved with the regimen with longer T > MIC. This was further confirmed in the experiments where the reference strain was exposed to amoxicillin/clavulanic acid for 100% of the experimental time, which gave a complete kill of the bacteria.

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REFERENCES

1. Eagle H, Fleischman R, Musselman AD. Effect of schedule of administration on the therapeutic efficacy of penicillin. *Am J Med* 1950; 9: 280–99.
2. Craig WA, Andes D. Pharmacokinetics and pharmacodynamics of antibiotics in otitis media. *J Pediatr Infect Dis* 1996; 15: 255–9.

3. Craig WA. Pharmacokinetic/Pharmacodynamic parameters: Rationale for antibacterial dosing of mice and men. *Clin Infect Dis* 1998; 26: 1–12.
4. Dagan R, Klugman KP, Craig WA, Baquero F. Evidence to support the rationale that bacterial eradication in respiratory tract infection is an important aim of antimicrobial therapy. *J Antimicrob Chemother* 2001; 47: 129–40.
5. Todd PA, Benfield P. Amoxicillin/clavulanic acid: an update of its antibacterial activity, pharmacokinetic properties and therapeutic use. *Drugs* 1990; 39: 264–307.
6. Kaye C, Allen A, Perry S *et al.* The clinical pharmacokinetics of a new pharmacokinetically enhanced amoxicillin/Clavulanic acid formulation. *Clin Therapeut* 2001; 23: 578–84.
7. Cars O, Henning C, Holm SE. Penetration of ampicillin and dicloxacillin into tissue cage fluid in rabbits: Relation to serum and tissue protein binding. *Scand J Infect Dis* 1981; 13: 69–74.
8. Löwdin E, Odenholt I, Bengtsson S, Cars O. Pharmacodynamic effects of sub-MICs of benzylpenicillin against *Streptococcus pyogenes* in a newly developed in vitro kinetic model. *Antimicrob Agents Chemother* 1996; 40: 2478–82.
9. Löwdin E, Odenholt I, Cars O. In vitro studies of pharmacodynamic properties of vancomycin against *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Antimicrob Agents Chemother* 1998; 42: 2739–44.
10. Vogelman B, Gudmundsson S, Turnidge J, Legget J, Ebert S, Craig WA. Correlation of antimicrobial pharmacokinetic parameters with therapeutic efficacy in an animal model. *J Infect Dis* 1988; 158: 831–47.
11. Cars O. Efficacy of beta-lactam antibiotics: integration of pharmacokinetics and pharmacodynamics. *Diagn Microbiol Infect Dis* 1997; 27: 29–33.
12. Andes D, Craig WA. In vivo activities of amoxicillin and amoxicillin-clavulanate against *Streptococcus pneumoniae*: application to breakpoint determinations. *Antimicrob Agents Chemother* 1998; 42: 2375–9.
13. Thorburn CE, Moleworth SJ, Sutherland R, Rittenhouse S. Postantibiotic and post β-lactamase inhibitor effect of amoxicillin plus clavulanate. *Antimicrob Agents Chemother* 1996; 40: 2796–2801.